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ABSTRACT

Objectives: This study aimed at determining the synergistic effects of Yuanhu Zhitong tablets (YHZTP) on alcohol- induced conditioned place preference (CPP) in mice, in addition, the intervention mechanism was preliminarily explored based on traditional Chinese Medicine (TCM) network pharmacology on alcohol addiction.

Methods: Alcohol-induced CPP mice were used to evaluate the effects of either YHZTP or levo-tetrahydropalmatine (*l*-THP) plus imperatorin (IMP) administration on animal behavior. The network pharmacological strategy was used to establish the "compound-target" and "disease-drug-target" network. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed on the shared targets between the compound and the disease. Twelve algorithms on CytoHubba were used to find the hub genes that were verified by qPCR.

Results: Systemic administration (2 g/kg, i.p.) of ethanol (EtOH) to mice was used to induce CPP. YHZTP On its own did not induce CPP or conditioned place aversion (CPA) at the doses of 0.3 g/kg or 0.6 g/kg (i.g.), but attenuated the acquisition and expression of EtOH-induce CPP in mice. In addition, YHZTP (0.3 or 0.6 g/kg) did not exhibit any effect on the motor activity of mice. Acquisition of alcohol-induced CPP was blocked by a combination of *l*-THP (5 mg/kg, i.g.) + IMP (2.5 mg/kg, i.g.) or *l*-THP (10 mg/kg, i.g.) + IMP (5 mg/kg, i.g.). However, the combination of *l*-THP (2.5 mg/kg, i.g.) + IMP (1.25 mg/kg, i.g.) or mono-administration of *l*-THP and IMP did not exhibit any effect on alcohol-induced CPP. YHZTP was also shown to reverse the up-regulation of Gabra1, Ptgs2, Mapk1, Mapk8, Mapk14, Nr3c, Prkca and Sirt1 genes and the down-regulation of Hhtr2a and Drd2 genes in the prefrontal cortex of EtOH induced CPP mice. These genes were associated with neuroactive ligand-receptor interactions, activation of the sphingolipid, calcium, cAMP, ErbB, NF-kappa B and MAPK signaling pathways.

Conclusion: YHZTP inhibits EtOH-induced CPP behavior in mice while a combination of *l*-THP and IMP exerts a synergistic effect on the reduction of EtOH-induced CPP. Possible pharmacological mechanisms include inhibition of the expression of inflammatory factors and regulation of neurotransmitter receptor levels. Therefore, YHZTP is a novel candidate for the treatment of alcohol addiction.

1. Introduction

In addition to opioids and mental substances, alcohol is an important addictive drug that easily induces toxic hallucinations. It exerts serious negative effects to the society and family. Globally, alcohol is among the most abused substances [1]. There has been no significant progress in the treatment of alcohol addiction within the last two years. Intervention mechanisms rely on adjuvant therapy. Currently, there is no clinical option that hinders withdrawal from alcohol dependence and prevention. Disulfiram, naltrexone, acamprosate and topiramate, with varying degrees of adverse reactions, are the main therapeutic options for alcohol addiction [2]. Chinese medicine is an effective and safe alternative for the treatment of alcohol addiction.

Since 1985, YHZTP was primarily composed of *Rhizoma corydalis* (yanhusuo) and *Angelica dahurica* (Baizhi) but was later improved with various editions of Chinese Pharmacopoeia [3,4]. It is a TCM preparation with analgesic, sedative and anti-inflammatory effects. Tetrahydropalmatine (*l*-THP) is one of the main components of *Rhizoma corydalis* in YHZTP that has been shown to be effective against a range of drug addictions [5–7]. However, given that YHZTP is a classic prescription, it has not been established whether it is effective for treating alcohol dependence. It has been reported that opioids induce glial cells to secrete inflammatory factors, activate natural immune signaling pathways, and enlarge the excitability of neurons involved in the development of drug addiction [8]. However, it is not clear if YHZTP exerts a therapeutic effect against alcohol addiction by inhibiting the expression of inflammatory factors.

The classical CPP model was used in this study to characterize the effects of YHZTP, *l*-THP (the main component of yanhusuo), IMP (the main component of Baizhi) and a combination of *l*-THP & IMP on alcohol-induced CPP in mice. CPP is a classical animal model of mental dependence that has been extensively used in the prediction and evaluation of drug dependence in studies [9,10].

The limitations associated with imprecise effective ingredients and the mechanisms of TCM compounds are documented. Pharmacology can be integrated with molecular networks that include multidisciplinary concepts such as biochemistry, bioinformatics and systems biology [11]. For this reason, this study

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utilized network pharmacology to explore the primary mechanisms of YHZTP. Studies have emphasized that the synergistic mechanisms of TCM should be clarified from the molecular network level [12,13].

Alcohol addiction is associated with impaired prefrontal cortex (PFC) functions. Therefore, the PFC of mice were selected for qPCR validation of the hub genes that had been predicted by network pharmacology. This was done to uncover the underlying synergistic mechanisms of YHZTP in treating alcohol addiction.

2. Materials and methods

2.1. Animals

Healthy male C57BL/6 mice (8–10 weeks old and weighing 24− 30 g) were purchased from the Laboratory Animal Center of Shantou University. They were housed in cages (4 mice per cage) under a 12 h/12 h light/dark cycle at a temperature of 24 ± 2 ℃ and a relative humidity of 30–70 %. Mice were provided with food and water ad libitum. Before the CPP behavior test, mice were acclimatized by being kept in the behavioral testing room for a week. All experiments were performed according to the animal protocols as approved by the Institutional Animal Care and Use Committee of Shantou University Medical College. Measures that minimized distress to the animals were put in place.

2.2. Drugs

YHZTP was purchased from the Sinopharm Group (Foshan) Dezhong Pharmaceutical Co., LTD. Prior to YHZTP being ground into a powder using a mortar, its coating was removed. CMC-Na (Carboxymethyl cellulose, CAS: 9004-32-4) (0.3 %) was then dissolved in distilled water and added to make a YHZTP suspension. A YHZTP dose of 0.3 g/kg (inferred from the human dose range using the formula: murine dose = 9.1 × human clinical dose) was administered to the mice [14]. In addition, the intragastric doses of YHZTP used in this study were 0.15 g/kg, 0.3 g/kg and 0.6 g/kg (0.1 ml/10 g). Alcohol was purchased from Sigma-Aldrich (USA). Ninety-five % of the alcohol was diluted with 0.9 % saline to 20 %. Thereafter, the mice were intraperitoneally (i.p.) administered with 20 % alcohol at a dose of 2 g/kg [15,16]. *l*-THP and IMP were purchased from ChemMall (Shanghai Baishun Biological Technology co., LTD) and a combination of both drugs administered at a ratio of 2:1 [17,18].

2.3. CPP apparatus

The CPP apparatus (Taimeng. China) consisted of two equal-sized compartments (20 cm × 20 cm × 15 cm) and one gray compartment (7.5 cm × 20 cm × 15 cm). The two equal-sized compartments were equipped with different visual and tactile cues. One was black with a gridded floor while the other was white with a round hole on the floor.
The time spent by the mice in each compartment as well as the distance covered were recorded using an infrared monitoring system.

2.4. CPP procedure

Alcohol-induced CPP was examined through a three-phase procedure as previously described [19] with some modifications (Fig. 1).

Pre-test phase (Days 1–3): mice were placed in the drug-free middle gray compartment. The doors were opened and the mice allowed to freely explore the two compartments for 20 min (Fig. 1A). The preferred compartment was defined as the one in which the mice spent a longer time while the other one was defined as the non-preferred compartment. The 3-day average time was taken as the baseline. Mice that spent over 850 s of the 20 min in either compartment and those that spent more time in the white compartment compared to the black one were excluded (6 out of 108) from the study. In this experiment, most mice preferred the black compartment, therefore, the experimental design was biased. Drugs or alcohol was paired with the white compartment in this study.

Conditioning phase (Days 4–11): all groups were administered with a saline injection and confined in the black compartment for 30 min (Day 4, 6, 8, 10). The control group was administered with saline while the alcohol and treatment groups were administered with an alcohol injection. The mice were confined in the white compartment for 30 min (Day 5, 7, 9, 11). Each mouse was subjected to four conditioning sessions (Fig. 1B).

Post-test phase (Days 12): The mice were allowed to freely explore the two compartments for 20 min without being gavaged or injected (Fig. 1C).

Behavioral conditioning training and testing was performed during the light phase of the light/dark cycle (8 am-6 pm). In addition, the mice were handled daily for a week before the behavioral experiment to help them adapt and minimize stress. They were then kept in a behavioral test room until the end of the CPP behavioral experiment. During the experimental procedures, a consistent temperature, humidity and smell were maintained in the behavioral test room. The entire CPP experiment was performed by the same person.

2.4.1. Alcohol addiction and the YHZTP therapeutic experiment The effects of alcohol in mice were induced by 2 g/kg of alcohol.

2.4.1.1. Effects of YHZTP on the acquisition of alcohol-induced CPP. Mice were distributed into five groups: the control group (water + saline); the alcohol group (water + alcohol) and the YHZTP treatment groups (YHZTP 0.15, 0.3 and

0.6 g/kg + alcohol, respectively). During the conditioning phase, 0.15, 0.3 and 0.6 g/kg YHZTP doses were gavaged, 30 min before alcohol injections.

2.4.1.2. Effects of YHZTP on the expression of alcohol-induced CPP. Mice were distributed into four groups: the control group (saline + water); the alcohol group (alcohol + water) and the YHZTP treatment groups (alcohol + YHZTP 0.3 and 0.6 g/kg, respectively). Mice were administered with YHZTP after the development of alcohol-induced CPP. During the Post-test (Day 12) phase, alcohol induced CPP in mice was evaluated without YHZTP treatment. During Post-test 1 (Day 13) and Post-test 2 (Day 14) the mice were administered with 0.3 g/kg and 0.6 g/kg YHZTP doses. Behavioral tests were performed 30 min after the administration. The respective treatments given to the mice are shown in Table 1.

Alcohol	Water	Saline	Alcohol	Water
Water				
YHZTP 0.3 g/kg	Water	Saline	Alcohol	Water
YHZTP 0.3				
g/kg				
YHZTP 0.6 g/kg	Water	Saline	Alcohol	Water
YHZTP 0.6				
g/kg				

i.g. intragastric administration; i.p. intraperitoneal injection.

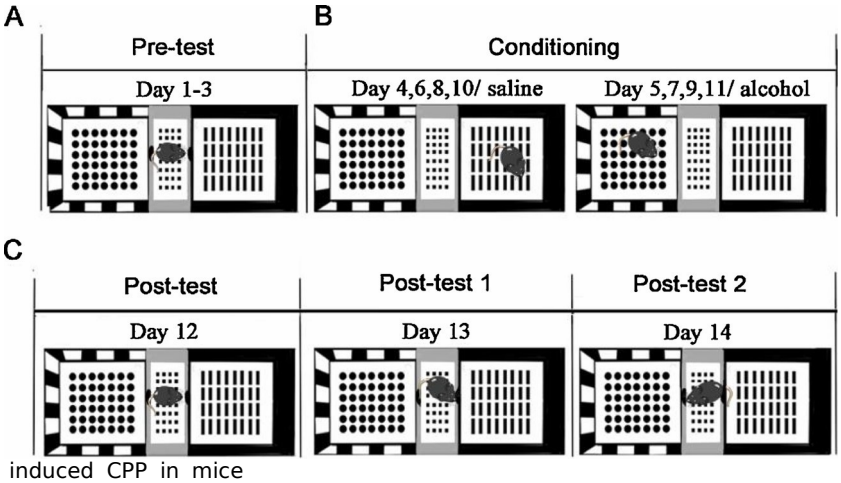


Fig. 1. Conditioned Place Preference (CPP) behavioral paradigm and testing schedule. (A) Pre-test phase (Days 1-3); (B) Conditioning phase (Days 4-11); (C) Post-test phase (Days 12,13,14).

2.4.2. Effects of different doses of YHZTP alone on place conditioning

Mice were distributed into three groups: the control group that was administered with water and the YHZTP groups that were administered with 0.3 and 0.6 g/kg of the drug, respectively. At days 4, 6, 8 and 10, the three groups were administered with an i.g. water gavage. After 30 min post-administration, they were confined in the black compartment for 30 min. At days 5, 7, 9 and 11, the YHZTP groups were gavaged with different drug doses (0.3 g/kg, 0.6 g/kg) and after 30 min, they were confined in the white compartment for 30 min.

2.4.3. Effects of combined l-THP and IMP on the acquisition of alcohol- induced CPP

Mice were distributed into seven groups: the control group (water + saline); the alcohol group (water + alcohol); the *l*-THP treatment group (*l*-THP 5 mg/kg + alcohol), the IMP treatment group (2.5 mg/kg + alcohol) and the *l*-THP + IMP treatment group (*l*-THP 2.5 mg/kg + IMP 1.25 mg/kg + alcohol; *l*-THP 5 mg/kg + IMP 2.5 mg/kg + alcohol; *l*-THP 10 mg/kg + IMP 5 mg/kg + alcohol). Pretreatment with *l*-THP, IMP, or *l*- THP + IMP was done by gavage 30 min before alcohol injections.

2.4.4. Effect of YHZTP on the distance covered by mice

The same CPP procedure as the one in section 2.4.2 was used. The infrared monitoring system in the CPP Apparatus was used to record the total distance covered by the mice during the pre- and post- tests.

2.5. Sample preparation

After the behavioral tests had been finalized, the mice were euthanized using isoflurane. Cardiac perfusion was performed using normal saline after which the whole brain was rapidly

Table 1
Experimental study design for the effects of YHZTP on the expression of alcohol- induced CPP.

Groups (n mice/group) = 6	Pre-test phase (i.g.) Day	Conditioning phase Treatment given at Day (Days i.g.)	(Days i.p.)	Post-test phase (i.g.) Day
	1,2,3 13,14	4,6,8,10	5,7,9,11	12
Control	Water	Saline	Saline	Water
Water				

harvested. The PFC [20, 21] was then dissected on ice and stored at - 80 °C for qPCR analysis.

2.6. Network pharmacology

2.6.1. Database building

Data on the two herbs in YHZTP were primarily obtained from the TCM systems pharmacology database (TCMSP, http://tcmspw.com/tcmsp.php) [22] and the China National Knowledge Infrastructure (CNKI). The Wanfang and PubMed databases were also used to supplement any other omitted components. The screening criteria included Oral Bioavailability (OB) ≥30 %, Drug-likeness (DL) ≥0.18, Caco-2 permeability ≥0 and the Blood Brain Barrier (BBB) ≥0.3 [23]. Volatiles that met the above criteria were selected as candidate compounds.

Data on YHZTP targets was obtained from four databases: TCMSP, Swiss Target Prediction (http://swisstargetprediction.ch/), PharmMapper (http://lilab-ecust.cn/pharmmapper/, updated on Nov. 27, 2017) and TargetNet (http://targetnet.scbdd.com/). Afterwards, we obtained the target proteins for the YHZTP bioactive components. Finally, the YHZTP target proteins were narrowed down to "Homo sapiens" proteins, using UniProt Knowledgebase (UniProtKB, https://www.uniprot.org/help/uniprotkb), to establish the "component- target" network.

Moreover, data on alcohol addiction-related targets was obtained from four databases: The Online Mendelian Inheritance in Man (OMIM, http://omin.org/), Human Genes|Gene Database|Gene Search (GeneCards, https://www.genecards.org/), a database of gene-disease associations (DisGeNET, http://www.disgenet.org/) and NCBI.

2.6.2. Screening for common targets of YHZTP and Alcohol addiction

Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn /) were used to determine the common targets for YHZTP and alcohol addiction.

2.6.3. Enrichment analysis of GO and KEGG pathways

The DAVID online analysis tool (https://david.ncifcrf.gov/) was used for GO enrichment analysis and KEGG pathway enrichment analysis of the common targets. Consequently, the targets and main pathways involved in YHZTP activities against alcohol addiction were obtained.

2.6.4. Protein-protein interaction networks and hub genes

A Protein-protein Interaction (PPI) network was constructed using the String Database (https://string-db.org/). Using a minimum interaction score of 0.9, the obtained data was imported into Cytoscape version 3.6.0. Additionally, 12 CytoHubba algorithms [24] were used to select the first 15 nodes for finding the hub genes.

2.7. Total RNA isolation

Brain tissue (15− 20 mg) was placed in a mortar and ground into powder using liquid nitrogen. Total RNA was isolated using the RNAeasy™ RNA extraction kit (Beyotime, Shanghai, China). The purity and quantity of the extracted RNA were determined using the Nanodrop 2000 spectrophotometer (Implen, Los Angeles, USA) and agarose gel electrophoresis.

2.8. Real-time quantitative PCR analysis

Based on the network pharmacology results, genes were selected for verification and primers designed using Primer Premier 5 software (supplementary Table 1). The RNA were reverse transcribed to cDNA using a cDNA Synthesis SuperMix qPCR kit (Transgen, Beijing, China). The cDNA was then amplified using an RT-PCR amplification kit (PerfectStart Green qPCR SuperMix) (Transgen, Beijing, China). The $2^{-\Delta\Delta Ct}$ method was used to perform the analysis while β-actin was utilized as the reference gene.

2.9. Statistical analysis

The CPP score was defined as the time spent in the non-preferred compartment during the post-test, post-test 1 or post-test 2 session minus the time spent in the non-preferred compartment during the pre- test session [25]. Statistical analyses were performed using the SPSS 24.0 software and data expressed as means ± SEM. One-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test were used to analyze the CPP and qPCR data. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of YHZTP on the acquisition of alcohol-induced CPP

The baseline pre-test time spent in the non-preferred compartment was similar among the control, alcohol and YHZTP (0.15, 0.3, 0.6 g/kg) treatment groups (Fig. 2A, one-way ANOVA, $F_{(4,25)} = 0.422$, $P = 0.791$). In addition, there was a significant increase in the post-test time spent in the non-preferred compartment by the alcohol group compared to the control (Fig. 2A, $^{**}p < 0.01$). The results also show that alcohol produced an obvious positional bias in the mice. Moreover, there was a significant difference between the alcohol and the YHZTP treatment groups with regard to the post-time spent in the non-preferred compartment ($F_{(3,20)} = 13.067$, $p < 0.001$). Post hoc analysis revealed that the time spent by the 0.15 g/kg YHZTP group was similar to that of the alcohol group, although there was a significant decrease in the 0.3 and 0.6 g/kg YHZTP groups (Fig. 2A, $p > 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$).

The alcohol group showed CPP on day 12 (Fig. 2B, $^{***}p < 0.001$ compared to the control group). There was a significant difference in the

CPP scores of the alcohol and YHZTP treatment groups ($F_{(3,20)}$ = 7.107, p < 0.01). Post hoc testing showed that the 0.15 g/kg dose of YHZTP did not exhibit a significant effect in preventing the acquisition of alcohol- induced CPP. However, the 0.3 g/kg and 0.6 g/kg YHZTP doses significantly attenuated alcohol-induced CPP (Fig. 2B, p >0.05, $^{\#}p$ <0.05 and $^{\#\#}p$ < 0.01 compared to the alcohol group, respectively).

3.2. Effects of YHZTP on the expression of alcohol-induced CPP

The baseline pre-test time spent in the non-preferred compartment was similar in both the control and alcohol groups (Fig. 3A, $F_{(1,22)}$ = 3.654, p > 0.05). However, there was a significant increase in the pre- test time spent in the non-preferred compartment by the alcohol group on Day 12 when compared to the control (Fig. 3A, ***p < 0.001). This implies that alcohol induced CPP in mice. Moreover, the CPP scores from the post-test revealed that alcohol induced the condition on day 12 (Fig. 3B, ***p < 0.001 compared to the control group).

On day 13, the alcohol group was further allocated into three groups: alcohol and the YHZTP (0.3, 0.6 g/kg) treatment groups. Water was administered to the control and alcohol categories while YHZTP (0.3, 0.6 g/kg, respectively) was gavaged to the YHZTP groups. The four groups were tested after 30 min. There was a significant increase in the post-test 1 time spent in the non-preferred compartment by the alcohol group compared to the control (Fig. 3C, ***p < 0.001). However, there was no significant difference in the post-time 1 spent in the non- preferred compartment between the alcohol and YHZTP treatment groups ($F_{(2,15)}$ = 2.024, p = 0.167). Nonetheless, the YHZTP treatment groups spent a shorter duration of time in the non-preferred compartment (Fig. 3C, p > 0.05 compared to the alcohol group). In addition, the CPP scores from the post-test 1 revealed that the YHZTP (0.3 g/kg, 0.6 g/ kg) did not exhibit any significant effect on the treated mice (Fig. 3D, p > 0.05 compared to the alcohol group).

On Day 14, all the groups were subjected to the same treatment as that on Day 13. There was a significant increase in time spent in the non- preferred compartment by mice from the alcohol group (Fig. 3C, **p < 0.01 compared to the control group). In addition, a decreasing trend in the time spent was observed in the 0.3 g/kg YHZTP treatment group compared to the alcohol group. Moreover, the results revealed a significant decrease in the non-preferred compartment time spent by the 0.6 g/kg YHZTP treatment group (Fig. 3C, $^{\#}p$ < 0.05 compared to the alcohol group). CPP scores from the Post-test 2 showed that the 0.3 g/kg YHZTP dose did not exhibit significant effects on the treated mice, however, the 0.6 g/kg dose of the drug significantly attenuated the

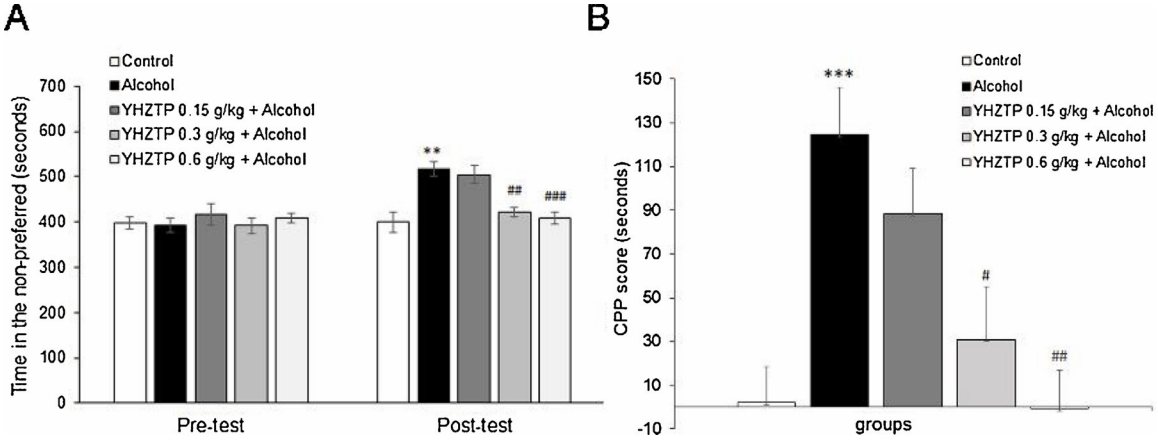


Fig. 2. Administration of 0.3 and 0.6 g/kg YHZTP attenuates the acquisition of alcohol-induced CPP. (A) Time spent in the non-preferred compartment before and after conditioning. (B) CPP scores. Data are represented as means ± SEM, ***p < 0.001, **p < 0.01 compared to the control group, $^{\#\#\#}p$ < 0.001, $^{\#\#}p$ < 0.01, $^{\#}p$ < 0.05 compared to the alcohol group, respectively. (N=6 per group).

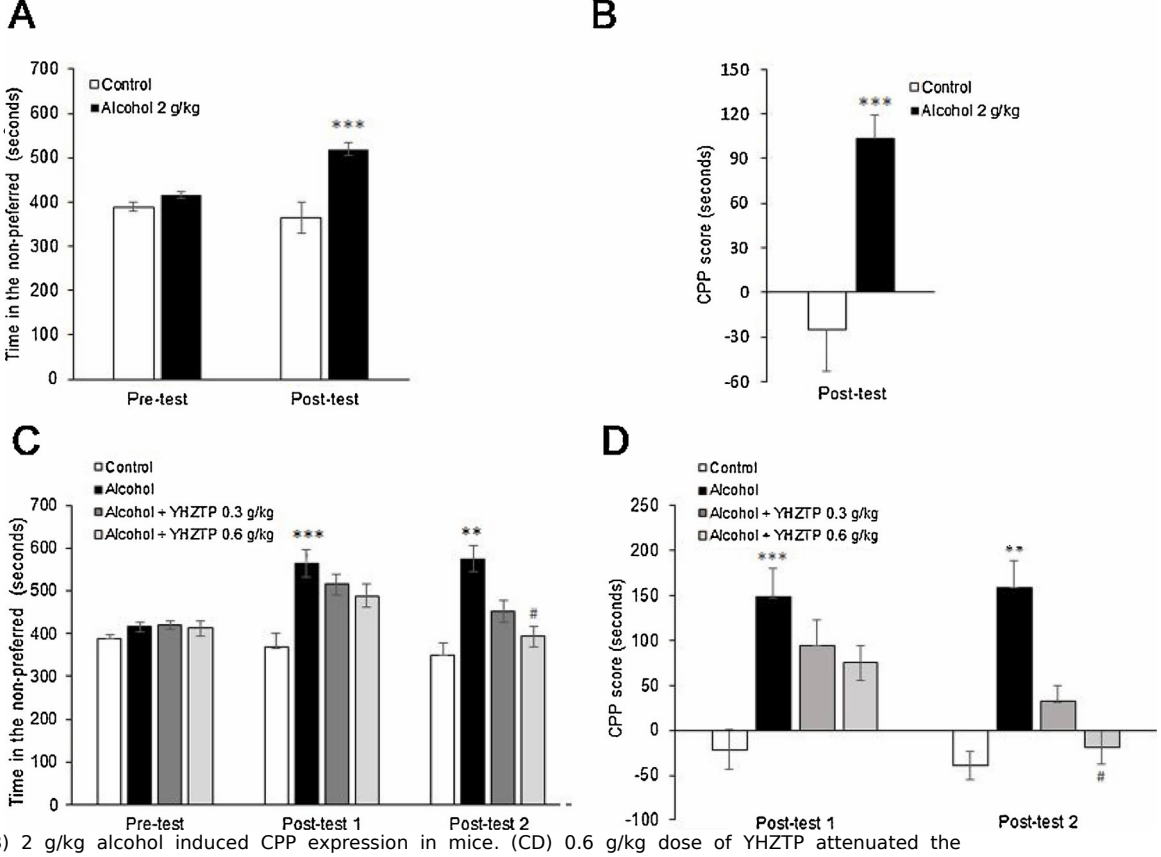


Fig. 3. (AB) 2 g/kg alcohol induced CPP expression in mice. (CD) 0.6 g/kg dose of YHZTP attenuated the expression of alcohol-induced CPP. (AC) Time spent in the non-preferred compartment. (BD) CPP scores. Data is presented as means ± SEM, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group, respectively. $^{\#}p < 0.05$ compared to the alcohol group. (N=6 per group).

expression of alcohol-induced CPP (Fig. 3D, $p > 0.05$, $^{\#}p < 0.05$ compared to the alcohol group, respectively).

3.3. Effects of different doses of YHZTP alone on place conditioning

The baseline pre-test time spent in the non-preferred compartment was similar in both the control and YHZTP (0.3 and 0.6 g/kg) groups (Fig. 4A, $F_{(2,15)} = 0.033$, $p = 0.968$). In addition, there was no significant difference in the post-test time spent in the non-preferred compartment among the three groups (Fig. 4A, $F_{(2,15)} = 0.451$, $p = 0.645$). CPP scores showed that there was no significant difference among the water control and YHZTP (0.3 and 0.6 g/kg) groups after the four conditioning sessions (Fig. 4B, $F_{(2,15)} = 0.427$, p=0.660). This suggests that YHZTP did not induce CPP or CPA by itself.

3.4. Effect of YHZTP on the exercise distance of mice

Fig. 5A shows the motion trajectories of the mice as revealed by the CPP infrared apparatus. The baseline pre-test distance covered by the water control and YHZTP (0.3 and 0.6 g/kg) groups was similar (Fig. 5B, $F_{(2,15)} = 1.029$, $p = 0.381$). In addition, the YHZTP testing doses were not able to influence the distance covered by mice in the post-test session (Fig. 5C, $F_{(2,15)} = 0.101$, $p = 0.905$). This suggests that the experimental doses of YHZTP alone (0.3, 0.6 g/kg) did not exhibit any effect on the motion activity of mice.

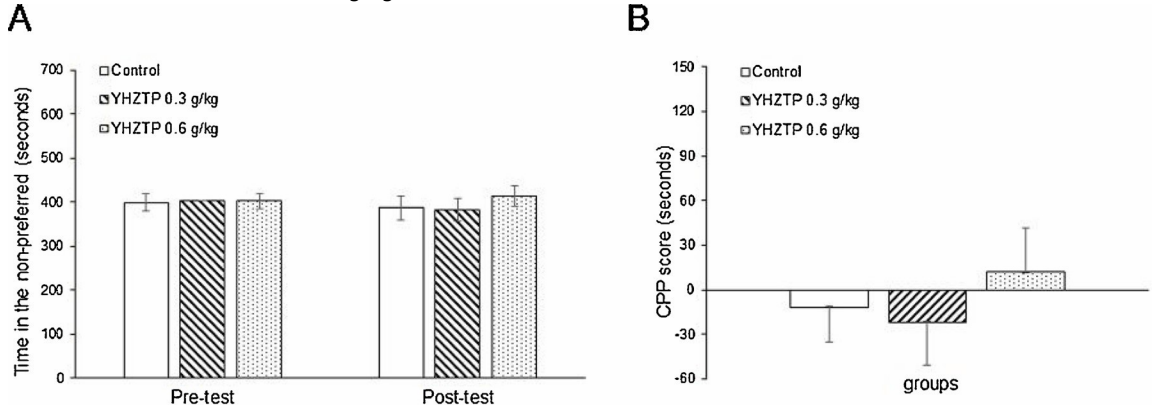


Fig. 4. YHZTP alone did not induce either CPP or CPA at the doses of 0.3 g/kg to 0.6 g/kg in mice. (A) Time spent in the non-preference compartment before and after conditioning. (B) CPP scores. Data are represented as means ± SEM. (N = 6 per group).

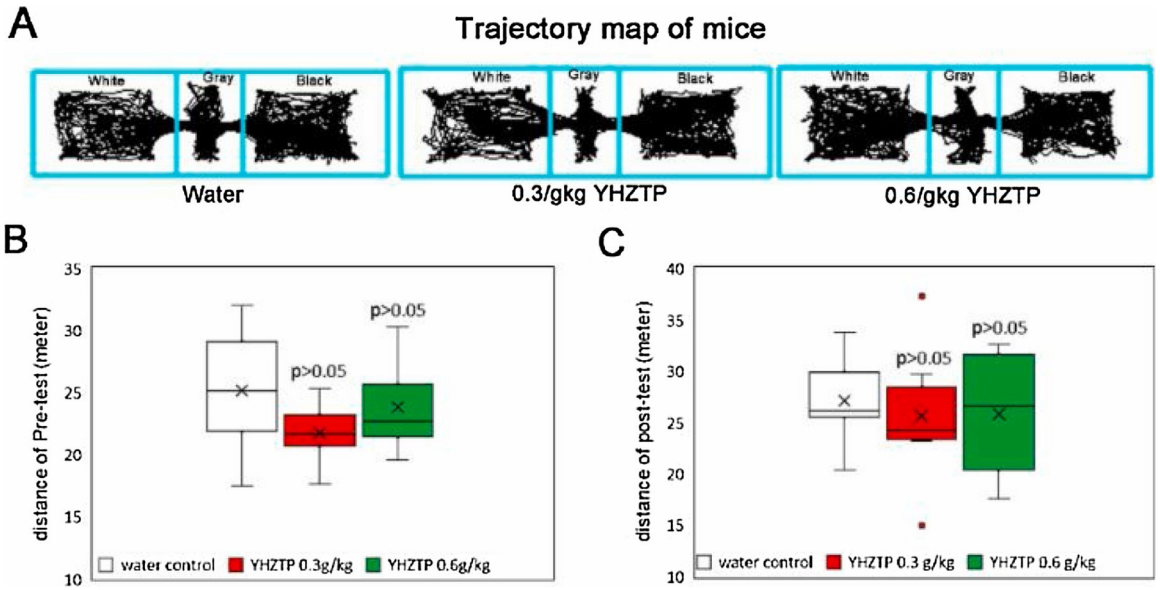


Fig. 5. YHZTP alone (0.3 and 0.6 g/kg) did not exhibit any effect on the motion activity of mice. (A) The motion trajectory of the mice. (B) Distance covered in the Pre-test. (C) Distance covered in the Post-test. Data are represented as means ± SEM. (N = 6 per group).

3.5. YHZTP component-target network

Herbal components were retrieved from four databases (Fig. 6A). A total of 301 components including 79 and 222 from two herbs; yanhusuo and Baizhi, respectively were obtained. The components were screened by four ADME related models: OB, DL, Caco-2 and BBB (Fig. 6B). Sixty bioactive components were included (Fig. 6C), 35 % of which (21/60) were from Baizhi while 65 % (39/60) were from yanhusuo (supplementary table 2).

Four disease gene target databases were used to screen for YHZTP- related targets (Fig. 6D). A total of 230 targets were screened. Yanhusuo had 1043 targets after excluding duplicates. One hundred and thirty- nine potential targets were eventually identified from 39 bioactive components. Additionally, Baizhi had 1097 targets after removing duplicates and 184 were finally identified. Notably, Yanhusuo and Baizhi had 94 common targets.

To understand the YHZTP component-target network from a systematic and holistic view, a network map was constructed using Cytoscape version 3.6.0 (Fig. 6E). The network contained a total of 293 nodes and 2077 edges. We found that multiple components acted on the same targets. For instance, DRD2 was a common target for hyndarin, cryptopine, corydaline, fumarine, canadine, glauvent, and isocorybulbine. GABRA1 was found to be a target for both yanhusuo and Baizhi.

3.6. Alcohol addiction-related targets and construction of the "disease- drug targets" network

A total of 927 targets were obtained from four disease gene target databases (Fig. 7A). After removing duplicates, a total of 687 alcohol addiction-related targets were obtained. Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were used to determine YHZTP and alcohol addiction target intersections. Notably, YHZTP was shown to share 89 common targets with alcohol addiction (Fig. 7B). An analysis of protein interactions was performed on the 89 common targets (Fig. 7C) and a "disease-drug-targets" network constructed using Cytoscape 3.6.0 (Fig. 7D).

3.7. Functional enrichment analysis

GO and KEGG pathway analyses were performed on the 89 common targets in order to establish the pharmacological mechanisms of YHZTP on alcohol addiction. GO analysis included Biological Process (BP, Fig. 8A), Cellular Component (CC, Fig. 8B) and Molecular Function (MF, Fig. 8C). The GO enrichment analysis results showed that the biological processes involved in the YHZTP mechanisms against alcohol addiction revolved around peptidyl-threonine phosphorylation, synaptic transmission, dopaminergic, negative regulation of I-kappaB kinase/NF- kappaB signaling and behavioral responses to cocaine (Fig. 8D). On the other hand, KEGG enrichment analysis showed that YHZTP may act on neuroactive ligand-receptor interactions as well as the sphingolipid, calcium, cAMP, FoxO, ErbB, NF-kappa B and MAPK signaling pathways (Fig. 8E).

3.8. Protein-protein interaction network and hub genes

The protein interaction network of the intersecting genes was obtained using String before importing into Cytoscape 3.6.0. The PPI network consisted of 89 nodes and 719 edges. Twelve algorithms on CytoHubba were used to find the hub genes shown in Fig. 9. Fifteen key

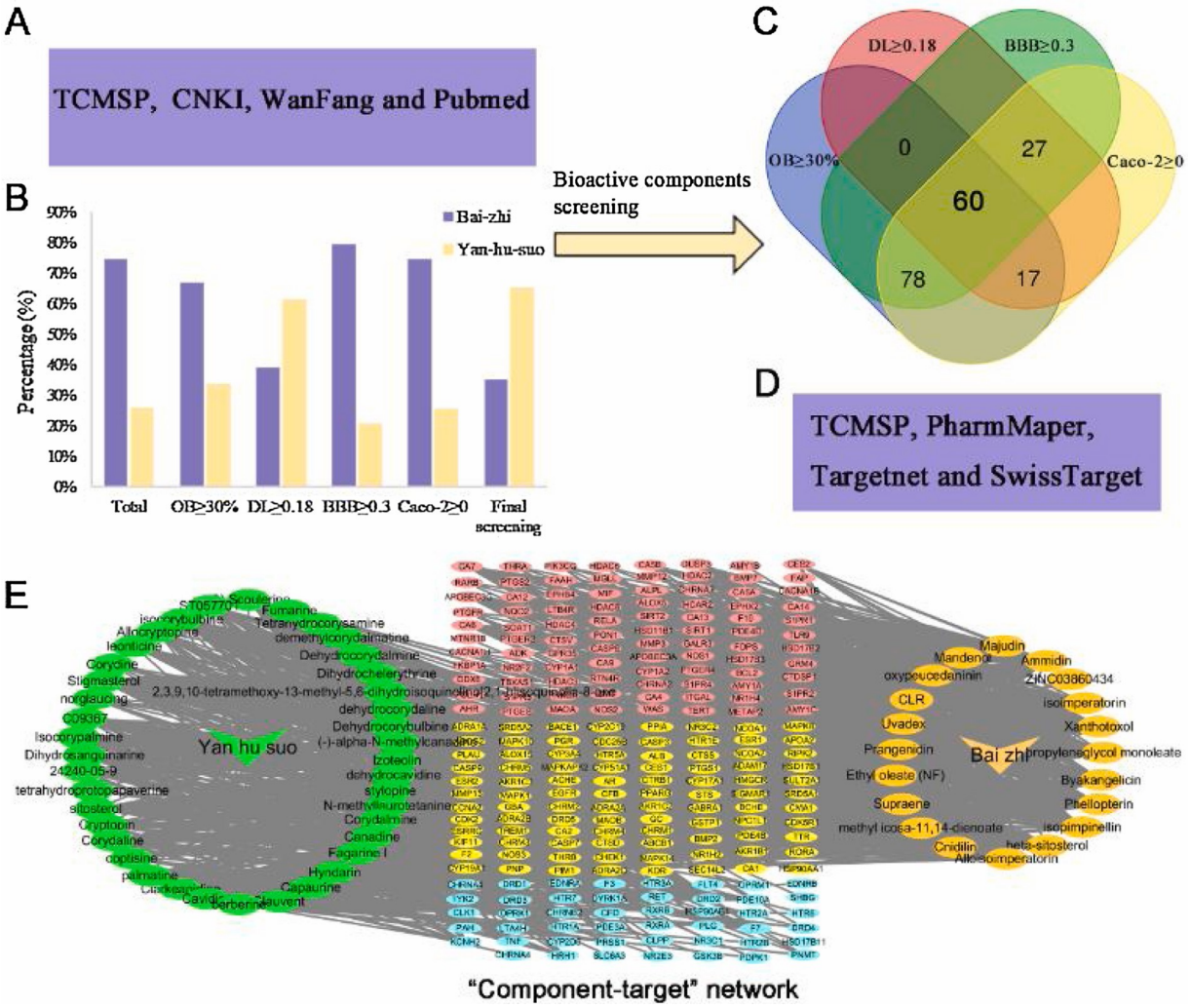


Fig. 6. The YHZTP component-target network. (A) Natural product databases and biomedical literature. (B) Distribution of different herbs. (C) 301 constituent- components and 60 bioactive components screened by four ADME-related models. (D) The four databases used for screening targets. (E) Construction of the putative YHZTP bioactive component-targets visual network. Green and orange represent the effective compounds of yanhusuo and Baizhi, respectively. Red and sky blue indicate the potential targets of yanhusuo and Baizhi, respectively. Light yellow represents the common potential targets (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

genes were finally identified: Alb, Htr2a, Casp8, Tnf, Rela, Gabra1, Ptgs2, Mapk1, Mapk8, Mapk14, Nr3c, Prkca, Sirt1, Drd2 and Ccna2. Some targets were exclusive to either yanhusuo or Baizhi while others were common to both.

3.9. Effects of combined l-THP and IMP on the acquisition of alcohol- induced CPP

Network pharmacology revealed that YHZTP exhibits a synergistic effect in the treatment of alcohol addiction. Therefore, *l*-THP and IMP were selected as the main components of YHZTP to verify this synergistic effect.

The baseline pre-test time spent in the non-preferred compartment was similar among the control, alcohol, *l*-THP, IMP and different doses of *l*-THP + IMP groups (Fig. 10A, $F_{(6,35)} = 0.565$, $p = 0.755$). Additionally, the post-test time spent by the alcohol group in the non- preferred compartment significantly increased compared to the control (Fig. 10A, $^{**}p < 0.01$). In addition, there was a significant difference among all the groups in the post-test time spent in the non-preferred compartment ($F_{(6,35)} = 4.747$, $p < 0.001$). Post hoc analysis showed that the time spent by the *l*-THP 5 mg/kg, IMP 2.5 mg/kg or combination of *l*-THP (2.5 mg/kg) + IMP (1.25 mg/kg) was similar to that of the alcohol group, however, combination of *l*-THP (5 mg/kg) + IMP (2.5 mg/kg) or *l*-THP (10 mg/kg) + IMP (5 mg/kg) significantly attenuated alcohol-induced CPP ($^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to the alcohol group, respectively, Fig. 10A)

CPP scores showed that the 5 mg/kg dose of *l*-THP, 2.5 mg/kg IMP or combined administration of *l*-THP (2.5 mg/kg) + IMP (1.25 mg/kg) did not exhibit any significant effect on the acquisition of alcohol-induced CPP. However, the combined administration of *l*-THP (5 mg/kg) + IMP (2.5 mg/kg), *l*-THP (10 mg/kg + IMP (5 mg/kg) significantly attenuated the acquisition of alcohol-induced CPP ($^{\#}p < 0.05$, $^{\#\#}P < 0.01$ compared to the alcohol group, respectively, Fig. 10B).

3.10. Confirmation of predicted hub gene expression using qPCR

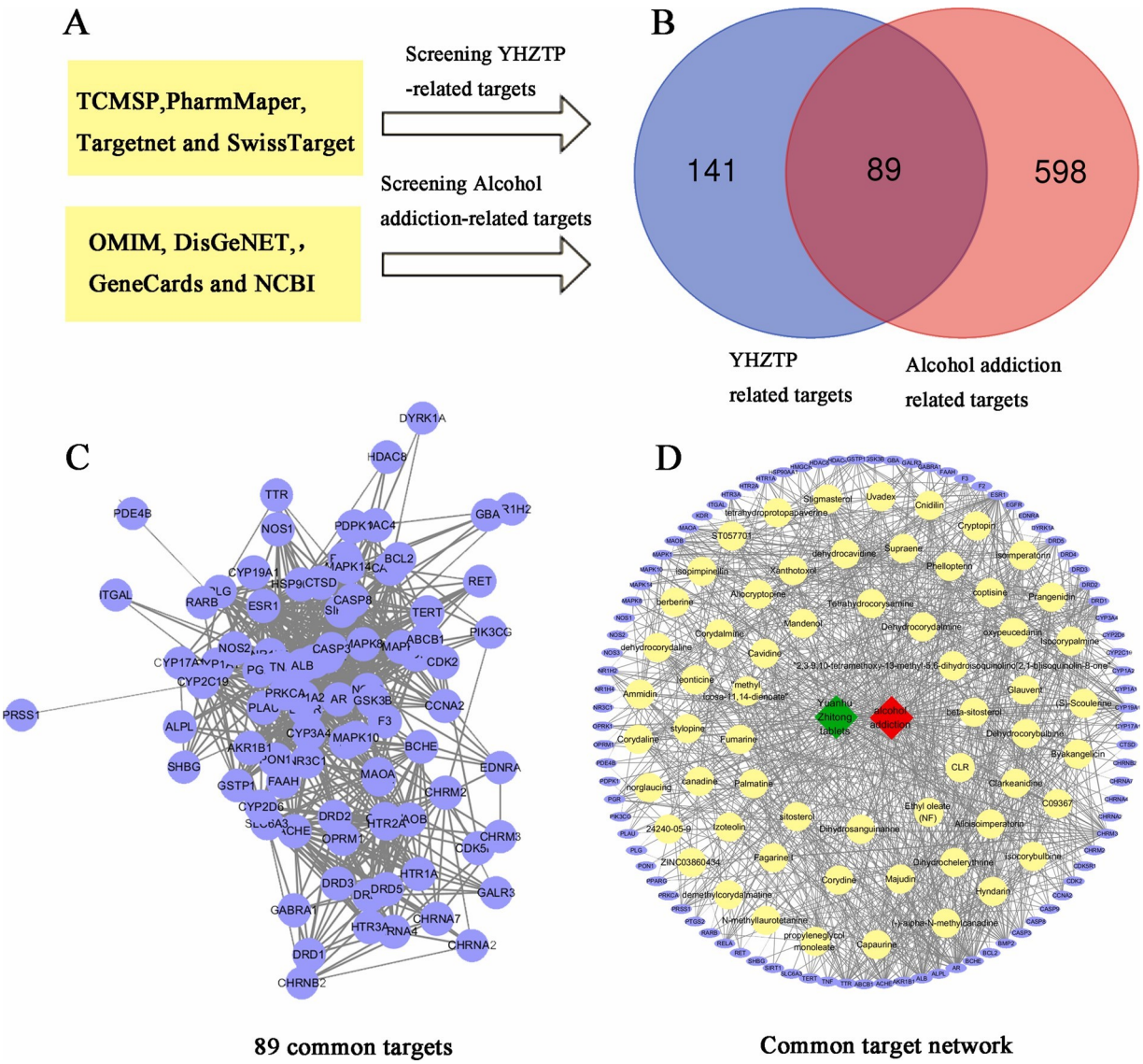
The relative expression of Htr2a and Drd2 in the alcohol group was low when compared to the control. However, expression of Gabra1, Mapk1, Mapk8, Mapk14, Nr3c, Prkca and Sirt1 was higher in the alcohol group than in the control group. Additionally, 0.6 g/kg YHZTP was able to reverse the alcohol-induced changes in the expression of these genes (Fig. 11).

4. Discussion

This study showed that exposure to a 2 g/kg dose of alcohol induces CPP in mice. However, a 0.3 or 0.6 g/kg YHZTP dose attenuates the acquisition of CPP. Furthermore, the 0.6 g/kg YHZTP dose was able attenuate CPP expression. These findings show that that pretreatment with YHZTP prevented the development of alcohol-induced CPP in mice. Administration of YHZTP after the development of alcohol-induced CPP could attenuate the condition. Therefore, we confirmed that YHZTP inhibited and reversed alcohol-induced CPP in mice. To the best of our knowledge, this is the first study that evaluates the therapeutic effects of YHZTP on alcohol addiction. The male mice were used in order to avoid the effects of the female estrus cycle on CPP.

Network pharmacology was used to find the active ingredients of YHZTP. Berberine, L-scoulerine, isocorypalmine (l-ICP), *l*-THP, dehydrocavidine (DEH), xanthotoxol, phellopterin and imperatorin (IMP) are all active components of YHZTP and may play an important synergistic role in the treatment of alcohol dependence. Berberine [26] plays a role in anti-meth addiction by modulating oxytocin receptors, drug preferences and anxiety-like behaviors. L-scoulerine [27] was shown to alleviate meth-induced behavioral changes in zebra fish and mice. Moreover, socorypalmine (l-ICP) [28] was reported to potentially act as a partial agonist to D1 and as a D2 antagonist in order to exert its effects *in vivo.* The drug may be a promising agent in the treatment of cocaine addiction. *l*-THP [29] attenuates the reinforcing and subjective properties of cocaine at doses that do not produce marked motor effects. This further emphasizes that *l*-THP may be beneficial for the management of cocaine addiction. Alcohol causes significant damage to the nervous system because it exerts a direct neurotoxic effect on the central nervous system, induces apoptosis in neurons, reduces the number of synapses and leads to organic changes in brain functions.

Fig. 7. "Disease-drug target" network. (A) Disease and YHZTP target databases. (B) There were 89 common targets between alcohol addiction and YHZTP. (C) Protein interactions among the 89 common targets. (D) The "Drug–disease–target" network. Pale yellow represents the 60 active components of YHZTP while blue represents the 89 common targets of YHZTP and alcohol addiction (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).



Dehydrocavidine (DEH) [30], imperatorin (IMP) [31], xanthotoxol [32] and phellopterin [33] do not have any reported anti-addictive properties. However, since they reduce oxidative damage, they can inhibit neuroinflammation and this may be beneficial in the treatment of alcohol addiction. In addition, previous studies showed that the essential oils or total coumarin of Baizhi combined with the total alkaloids of yanhusuo increased the absorption of *l*-THP [34,35]. Both *l*-THP and corydaline were reported to inhibit withdrawal symptoms and weight loss in morphine-dependent abstinence rats [36]. These compounds are active ingredients of YHZTP. This implies that the drug may have a multi-component synergistic effect in the treatment of alcohol addiction. In this study, the synergistic effect of *l*-THP and IMP was verified by the CPP experiment. The combined administration of *l*-THP and IMP significantly inhibited alcohol-induced CPP in mice.

Network pharmacology predicted that YHZTP attenuates alcohol addiction by regulating 10 targets (Htr2a, Drd2, Gabra1, Ptgs2, Mapk1, Mapk8, Mapk14, Nr3c, Prkca and Sirt1). We

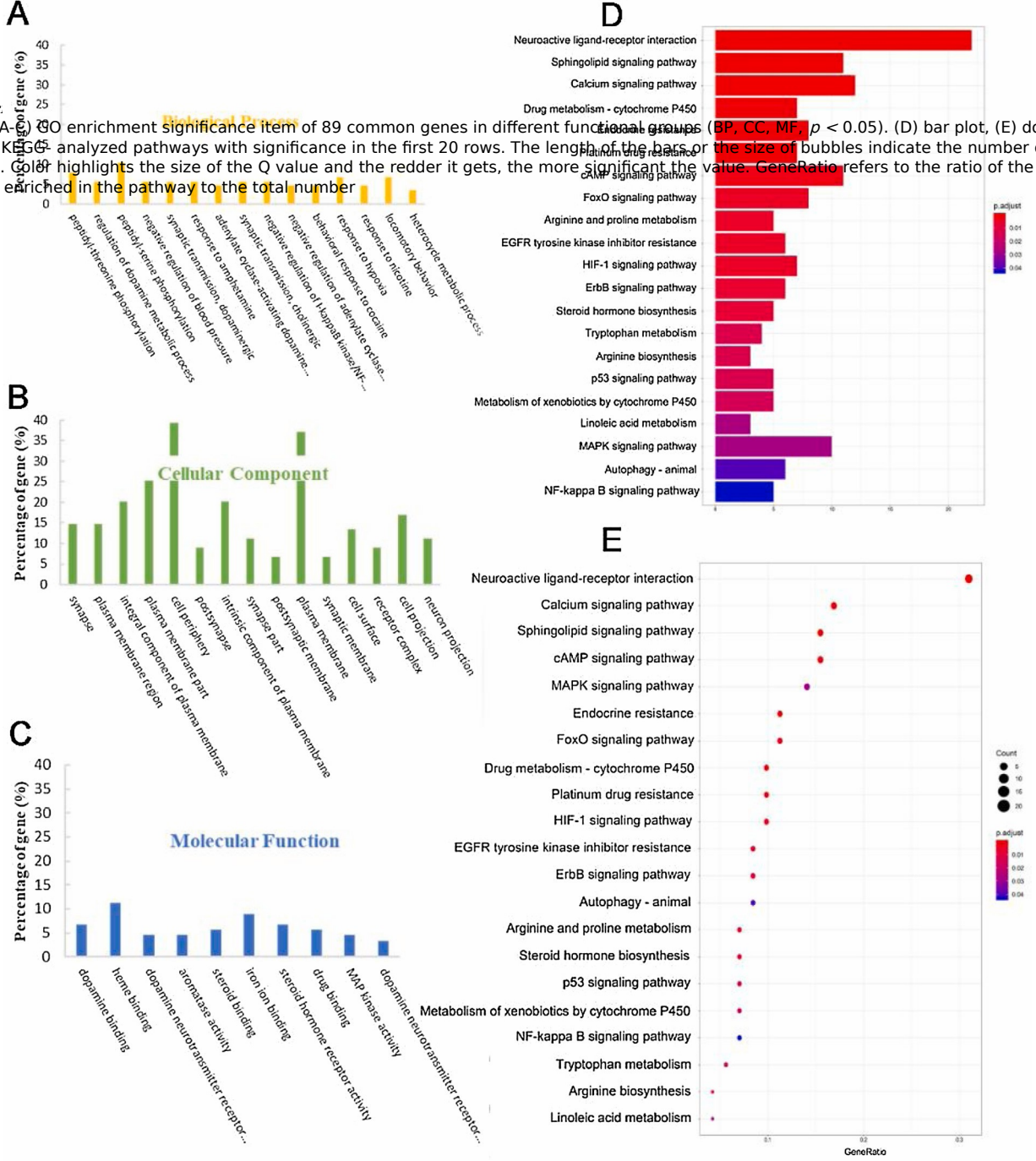


Fig. 8. (A-C) GO enrichment significance item of 89 common genes in different functional groups (BP, CC, MF, $p < 0.05$). (D) bar plot, (E) dot plot, $p < 0.05$). KEGG analyzed pathways with significance in the first 20 rows. The length of the bars or the size of bubbles indicate the number of genes enriched. Color highlights the size of the Q value and the redder it gets, the more significant the value. GeneRatio refers to the ratio of the number of genes enriched in the pathway to the total number of common genes (89).

found that alcohol causes genetic changes in the PFC of mice. It was also found that YHZTP reverses alcohol-induced changes in the expression of these genes. Targets and signaling pathways were closely related to inflammation and neurotransmitters. Among the genes, GABRA1 has been associated with a variety of neurosystemic diseases. Elevated GABRAl expression levels are associated with alcohol craving and severity in alcohol-dependent patients [37,38]. HTR2A receptors are widely distributed in the brain and abnormalities in their structure and function are associated with schizophrenia, depression, alcohol and heroin abuse [39,40]. Furthermore, DRD2 plays a critical role in morphine- [41] and cocaine-induced [42] addiction in rats. The expression of this gene was down-regulated in the PFC, NAC and VTA of morphine addicted [43,44]. In this study, alcohol up-regulated Gabra1 and down-regulated Drd2 and Htr2a in the PFC region. This suggests that YHZTP exerts its therapeutic effects against alcohol addiction by regulating neurotransmitter levels.

SIRT1 was shown to be involved in the regulation of heroin addiction in rats. SIRT1 gene silencing reduced withdrawal symptoms and CPP in rats addicted to the drug. However, SIRT1 overexpression increased the number of synapses in the NAC of rats [45]. In this study, we hypothesized that the up-regulation of Sirt1 in the PFC brain region may enhance the memory of alcohol-addicted mice, affect synaptic plasticity and contribute to the regulation of alcohol addiction.

Reports on the effects of neuroimmune molecules on neuronal plasticity and overall brain circuit functions are emerging [46]. Proinflammatory immune signals also have

neuroexcitatory effects. In the brain, these molecules are involved in immune functions and also play integral roles in normal brain development as well as behavior by modulating synaptic activity [47]. We speculated that immunoinflammatory molecules such as Mapk1, Mapk8 and Mapk14 in the Mapk signaling pathway are involved in the development of alcohol addiction by regulating neuron excitability and plasticity. In addition, YHZTP reduces alcohol-induced neuroinflammation and neuronal excitability

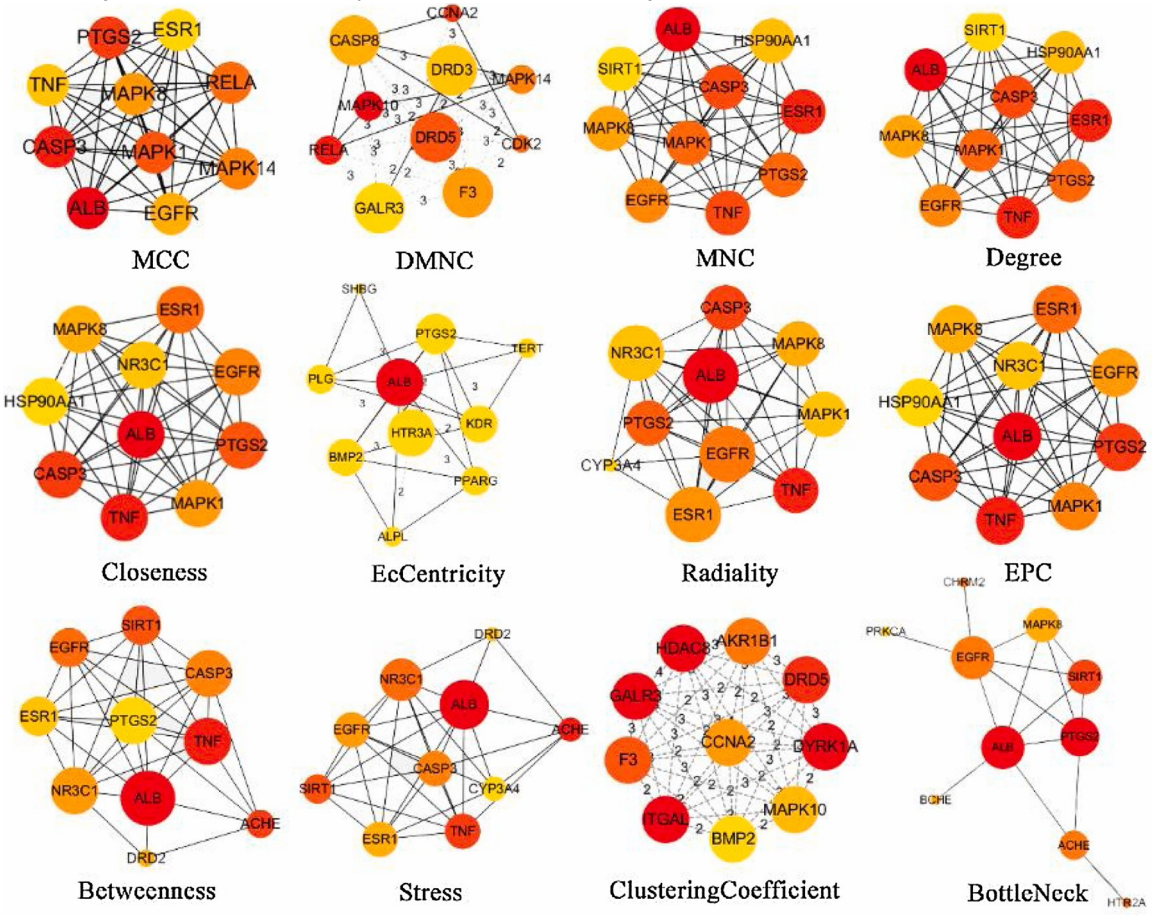


Fig. 9. Results of the algorithms from cytoHubba. The PPI network data from String was further analyzed using Cytoscape 3.6.0 and hub gene identification was performed by cytoHubba, based on 12 algorithms. PPI, Protein-protein Interaction. The circles represent the nodes while circle size indicates the degree.

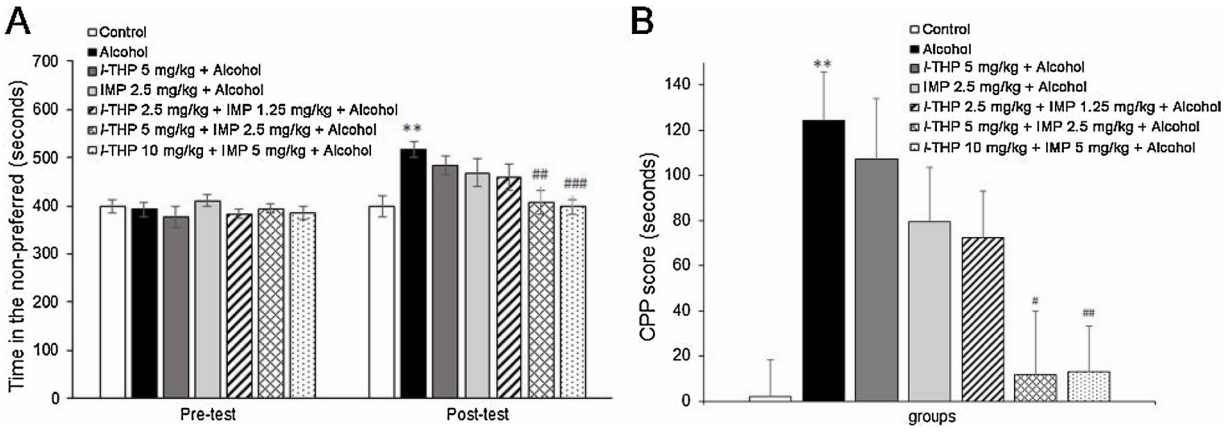


Fig. 10. Combined administration of *l*-THP (5 mg/kg) + IMP (2.5 mg/kg), *l*-THP (10 mg/kg) + IMP (5 mg/kg) could prevent the acquisition of alcohol-induced CPP. (A) The time spent by the mice in the non-preferred compartment. (B) CPP scores. Data are represented as means ± SEM, $^{**}p < 0.01$ compared to the control group, $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ compared to the alcohol group, respectively. (N=6 per group). by inhibiting the expression of proinflammatory factors, thereby, **Financial support and sponsorship** inhibiting alcohol intake.

5. Conclusion

YHZTP modulates the development and expression of alcohol-induced CPP. Therefore, the drug might be useful in the treatment of alcohol addiction.

Declaration of Competing Interest

The authors report no declarations of interest.

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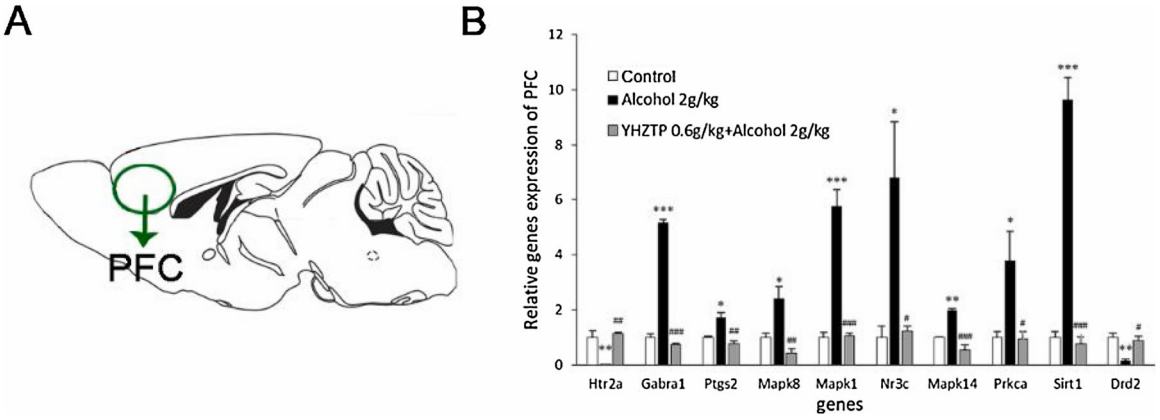

Fig. 11. (A) PFC in mice. (B) 0.6 g/kg YHZTP reversed alcohol-induced down regulation of Htr2a and Drd2 and enhanced the expression of Gabra1, Ptgs2, Mapk1, Mapk8, Mapk14, Nr3c, Prkca and Sirt1 in the PFC of mice. Data are represented as means ± SEM, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the control group, $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$ compared to the alcohol group, respectively. (N=3 per group).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110962.

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